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Regulation of protein synthesis: Activation by double-stranded RNA of a protein kinase that phosphorylates eukaryotic initiation factor 2

(initiation in reticulocyte lysates/translational control/Met-tRNA$_f^{Met}$)

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ABSTRACT **Incubation of reticulocyte lysates or isolated crude ribosomes with low levels of double-stranded RNA (0.1–10 ng/ml) induces the formation of an inhibitor of protein synthesis initiation similar to that observed in heme deficiency. The inhibitor is associated with a cyclic AMP-independent protein kinase activity (ATP:protein phosphotransferase, EC 2.7.1.37) that phosphorylates the small polypeptide (38,000 daltons) of the eukaryotic initiation factor eIF-2. Activation of the inhibitor requires ATP in addition to double-stranded RNA and is accompanied by the phosphorylation of a 67,000-dalton polypeptide of unknown function. The inhibitor remains associated with the ribosomes during high-speed sedimentation. Once formed, the ribosome-associated inhibitor phosphorylates eIF-2 and inhibits protein synthesis in the absence of double-stranded RNA. Inhibition is prevented by exogenous eIF-2. The bound inhibitor can be solubilized by extraction with 0.5 M KCl. The soluble inhibitor preparation retains the ability to phosphorylate the small polypeptide of eIF-2 and to inhibit protein synthesis. Untreated crude ribosomes also contain cyclic AMP-independent protein kinase activities that phosphorylate the middle polypeptide (49,000 daltons) of eIF-2 and several polypeptide subunits of eIF-3 (160,000, 125,000, and 65,000 daltons); these kinase activities are not affected by double-stranded RNA and do not inhibit protein synthesis.**

Initiation of protein synthesis in rabbit reticulocyte lysates is rapidly inhibited by low levels (0.1–10 ng/ml) of double-stranded RNA (dsRNA) (1, 2). The mechanism of inhibition is similar to that induced in lysates by heme deficiency (3). Both inhibitions are characterized by (*a*) biphasic kinetics (2, 3); (*b*) polyribosome disaggregation (2, 4); (*c*) depletion of the 40S ribosomal subunit–Met-tRNA$_f^{Met}$ complex (5, 6); (*d*) prevention by high cyclic AMP (cAMP) concentrations (1–10 mM) (7, 8), and potentiation by ATP (9, 10); (*e*) activation of a cAMP-independent protein kinase activity (ATP:protein phosphotransferase, EC 2.7.1.37) that phosphorylates the small polypeptide (38,000 daltons) of eukaryotic initiation factor 2 (eIF-2) (10—14), the initiation factor that binds Met-tRNA$_f^{Met}$; and (*f*) reversal by exogenous eIF-2 (15–17). However, in contrast to the activation of the heme-regulated inhibitor (HRI) that can occur in postribosomal supernates (18–20), activation of the dsRNA-induced inhibitor (dsI) is associated with the ribosomes (10). The activation of dsI requires dsRNA and ATP and is accompanied by the phosphorylation of a 67,000-dalton polypeptide whose function is not known; the activation of dsI results in the phosphorylation of the small polypeptide of eIF-2 (10). Similar dsRNA-dependent phosphorylation patterns and inhibitions of protein synthesis are observed in cell-free extracts derived from interferon-treated cells (21–23). In the present report, we examine several properties of dsI. Following activation of dsI on isolated crude ribosomes, the dsI activity can be sedimented with the ribosomes. The ribosome-associated dsI inhibits protein synthesis in lysates and is associated with a cAMP-independent protein kinase activity that phosphorylates the small polypeptide of eIF-2. Once dsI is activated, the phosphorylation of eIF-2 and the inhibition of protein synthesis are independent of dsRNA (10). The ribosome-associated inhibitor can be solubilized by extraction with 0.5 M KCl. Inhibition of protein synthesis by the ribosome-associated dsI [dsI(r)] or soluble dsI [dsI(s)] is specifically overcome by exogenous eIF-2. Untreated crude ribosomes also contain protein kinase activities that phosphorylate the middle polypeptide (49,000 daltons) of eIF-2, and several polypeptide subunits of eIF-3 (160,000, 125,000, and 65,000 daltons) (24); these phosphorylations do not appear to inhibit protein synthesis and are not affected by preincubation with dsRNA. It is of interest that of these various protein kinase activities, the phosphorylation of the 38,000-dalton polypeptide of eIF-2 by dsI(r), dsI(s), or partially purified HRI is selectively diminished in the presence of added hemin.

METHODS AND MATERIALS

The preparation of rabbit reticulocyte lysates has been described (25). Protein synthesis was determined by the uptake of [^{14}C]leucine in 25-μl or 50-μl reaction mixtures containing 50% lysate (vol/vol) as previously described (16, 25).

Induction and Assay of dsI Activity. The induction of dsI activity was followed by (*a*) the addition of *Penicillium chrysogenum* mycophage dsRNA directly to protein synthesis assays (see Figs. 1, 2*A*); or by (*b*) preformation of dsI in intact lysates (see Fig. 2 *B* and *C*) or on isolated crude ribosomes (see Figs. 3–5), followed by assays for dsI activity in standard protein synthesis reaction mixtures (10). For the isolation of crude ribosomes, 1 ml of a freshly thawed lysate was prewarmed (5 min at 37°), chilled, passed through a Sepharose 6B column (1 cm × 17 cm) (10) prewashed with 2 ml of lysate, and then equilibrated in 6B buffer [10 mM Tris·HCl (pH 7.6)/25 mM KCl/2 mM Mg(OAc)$_2$/5% (vol/vol) glycerol] (10) (see Fig. 3). Peak ribosome fractions (17 A_{260} units/ml; 1 *A* unit being the amount of material giving an absorbance of 1 when dissolved in 1 ml and the path length is 1 cm) were used directly for dsI formation. The activation and assay of dsI were carried out in 2 steps.

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Abbreviations: dsRNA, double-stranded RNA; dsI, dsRNA-induced inhibitor; dsI(r), ribosome-associated dsI; dsI(s), solubilized dsI; HRI, heme-regulated inhibitor; cAMP, cyclic AMP. Eukaryotic initiation factor designations (eIF-1, 2, 3, 4A, 4B, 4C, 5) (24) are those adopted at the Fogarty International Symposium on Protein Synthesis at the National Institutes of Health (1976, Bethesda, MD).

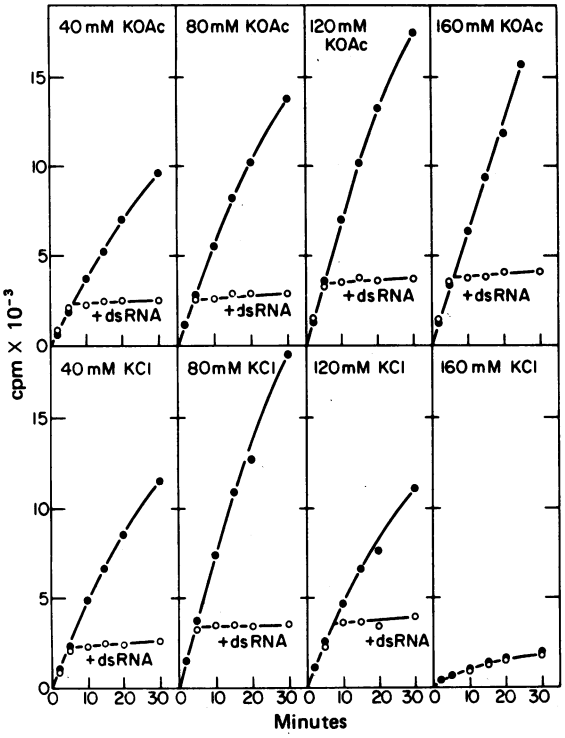

FIG. 1. Comparison of KCl and KOAc. Protein synthesis assays (50 μl) were carried out at 30° in the presence (○) and absence (●) of mycophage dsRNA (20 ng/ml) and KCl or KOAc as indicated. At intervals, 5-μl aliquots were assayed for protein synthesis.

Activation of dsI (step 1): Reaction mixtures contained 8 mM Tris·HCl (pH 7.8), 80 mM KCl, 4 mM NaCl, 2.6 mM $Mg(OAc)_2$, 1 mM unlabeled ATP, 4% glycerol, 80% lysate (vol/vol) or crude ribosomes at 13 A_{260} units/ml, and mycophage dsRNA at 20 ng/ml (or as indicated); incubation was 20 min at 37° except where indicated. *Assay of dsI* (step 2): Portions of step 1 reaction mixtures were assayed in 25-μl (or 50-μl) protein synthesis assays supplemented with 20 μM hemin and poly(I·C) at 20 μg/ml (where indicated). In general, a 1:10 dilution of *step 1* lysate dsI into step 2 assays produced a 60–80% inhibition of protein synthesis in 30 min at 30° (see Fig. 2*C*). The omission of dsRNA or ATP during dsI activation (step 1) yielded no inhibition in *step 2* incubations. Hemin had no effect on dsI formation in *step 1*.

Preparation of Particulate dsI and Soluble dsI. dsI(r), prepared on crude ribosomes (see above), was sedimented at 150,000 × *g* for 30 min, and resuspended in 6B buffer at 18 A_{260} units/ml. The suspension contained 75% of the initial dsI activity and could be stored at −80°. For dsI(s) (see Fig. 5), 300 μl of dsI(r) was extracted with 0.5 M KCl for 1 hr at 0°. After sedimentation, the extracted pellet was resuspended in 250 μl of 6B buffer (13 A_{260} units/ml); the supernate [dsI(s)] was concentrated to 1.5 mg/ml by dialysis against 20 mM Tris·HCl (pH 7.6), 100 mM KCl, 10% glycerol, and 22% (vol/vol) polyethylene glycol (Carbowax 6000, Union Carbide).

Protein Kinase Assays and Sodium Dodecyl Sulfate/Acrylamide Electrophoresis. Protein kinase assays (20 μl) contained 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.1), 60 mM KCl, 2 mM $Mg(OAc)_2$, and 125 μM [γ-^{32}P]ATP (2 Ci/mmol) as described (11); for other additions, see Fig. 6. Incubation was 10 min at 37°. Slab gel electrophoresis [0.1% sodium dodecyl sulfate/10% acrylamide/0.26% *N,N'*-methylene bis(acrylamide)], and autoradiography, have been described (11).

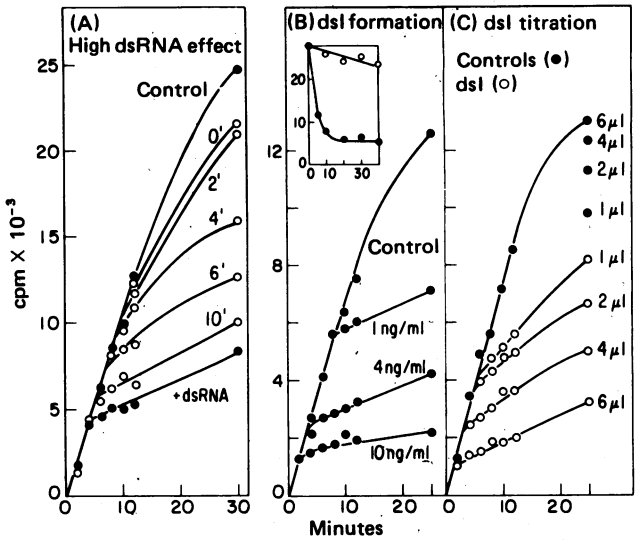

FIG. 2. Parameters of dsI formation in lysates. (*A*) Protein synthesis assays (50 μl) contained mycophage dsRNA (20 ng/ml) except in one control (●). At 0, 2, 4, 6, and 10 min, poly(I·C) (20 μg/ml) (○) was added to separate incubations as indicated; 5-μl aliquots were assayed at intervals for protein synthesis. (*B*) Dependency of dsI formation on dsRNA concentration was assayed in 2 steps. Step 1 activation assays with whole lysate (80% vol/vol) contained mycophage dsRNA (1, 4, or 10 ng/ml). After 15 min at 37°, 5-μl portions of each incubation were added to separate step 2 protein synthesis assays (25 μl) containing poly(I·C) at 20 μg/ml; 3 μl was assayed at intervals for protein synthesis. (*Inset*) Time course of dsI activation plus (●) and minus (○) mycophage dsRNA (20 ng/ml); at intervals, 5-μl aliquots were assayed for dsI in step 2 incubations (50 μl) containing poly(I·C) (20 μg/ml). (*C*) Lysate dsI preformed for 20 min at 37° plus (○) and minus (●) mycophage dsRNA (20 ng/ml) was titrated (1, 2, 4, or 6 μl) in step 2 protein synthesis assays (25 μl) containing poly(I·C) at 20 μg/ml; 3-μl aliquots were assayed at intervals. In some control assays, only the final time point is shown.

P. chrysogenum mycophage dsRNA was a gift of Hugh Robertson (Rockefeller University). eIF-2 (90% pure) was a gift of William C. Merrick (National Institutes of Health). Highly purified eIF-3, 4A, 4B, 4C plus 5, and partially purified eIF-1 and 2, were gifts of Hans Trachsel (Massachusetts Institute of Technology). [^{14}C]Leucine and [γ-^{32}P]ATP were obtained from New England Nuclear, and poly(I·C) from Sigma Chemical Co.

RESULTS AND DISCUSSION

Parameters of dsI Formation. Recent findings that high KOAc concentrations support protein synthesis in lysates more efficiently than comparable KCl levels (26) led us to examine the effects of these salts on the inhibition induced by dsRNA in lysates. Optimal protein synthesis was obtained by adding either 80 mM KCl or 120 mM KOAc (Fig. 1). At concentrations of 160 mM added salt, KOAc efficiently supported protein synthesis, whereas KCl was completely inhibitory; actual K^+ concentrations in the reaction mixtures were probably 10–30 mM higher due to endogenous KCl (26). These values are in accord with previous findings which indicated that high Cl^- levels are inhibitory (26). In the presence of *P. chrysogenum* mycophage dsRNA (20 ng/ml), biphasic kinetics of inhibition were obtained at all salt levels between 40 and 160 mM KOAc or 40 and 120 mM KCl.

An interesting property of the lysate system is its insensitivity to high dsRNA levels (1–20 μg/ml) (27). Because high dsRNA levels do not affect the inhibitory function of dsI once it is formed, this property provides a convenient probe to distinguish

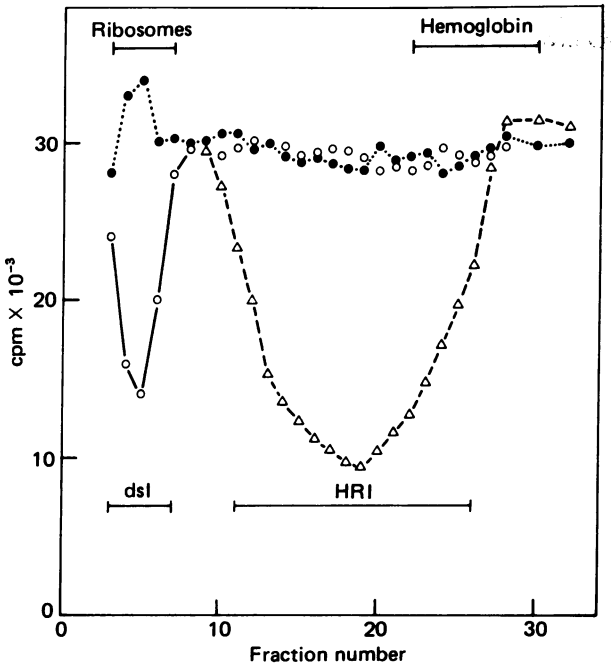

FIG. 3. Distribution of dsI and HRI in lysates fractionated on Sepharose 6B. Fresh lysate was fractionated in Sepharose 6B and each fraction was monitored for its capacity to form dsI (○) or HRI (△) as described in the text. Portions (5 μl) of each fraction in the activation profiles were added to separate step 2 protein synthesis assays (25 μl) containing poly(I·C) at 20 μg/ml; untreated aliquots of the Sepharose fractions (●) were similarly assayed. After 30 min at 30°, 5-μl aliquots were assayed for protein synthesis.

between dsI formation and dsI activity (10, 27). The delayed addition of poly(I·C) (20 μg/ml) to lysates supplemented at zero time with inhibitory levels of mycophage dsRNA (10 ng/ml) effectively blocks further dsI formation, but permits expression of the preformed dsI (Fig. 2A). As the addition of poly(I·C) is increasingly delayed, there is a corresponding increase in the extent of inhibition. By 10 min, most of the dsI potential of the lysate has been expressed, and subsequent addition of the high concentration of poly(I·C) has essentially no effect (Fig. 2A) (27). The activation of dsI (step 1) in intact lysates is dependent on ATP (data not shown) (10), the concentration of mycophage dsRNA (Fig. 2B), and time (Fig. 2B, *inset*). Under the conditions of activation, optimal formation of lysate dsI required a mycophage dsRNA concentration of at least 10 ng/ml. The extent of inhibition of protein synthesis (step 2) by lysate dsI was proportional to the amount of dsI added (Fig. 2C); comparable samples of a control lysate preparation (−dsI) produced corresponding stimulations of protein synthesis (Fig. 2C).

Association of dsI with Ribosomes. Farrell *et al.* (10) have demonstrated that dsI can be formed on isolated crude ribosomes. If fresh lysates are fractionated on Sepharose 6B and each fraction is monitored for its capacity to form dsI, all of the dsI activity is associated with the ribosomal fractions (Fig. 3). In the same fractionation profile, the proinhibitor form of the heme-regulated inhibitor (HRI) is retarded; distribution of the putative HRI was determined by activation of the proinhibitor fractions with *N*-ethylmaleimide (Fig. 3) (28). Untreated control fractions, similarly assayed, had no effect on protein synthesis. The same profile is obtained if dsI is preformed in intact lysates prior to fractionation on Sepharose 6B (10). Moreover, we find the amount of dsI activity formed on the isolated ribosomes is comparable to the amount of dsI formed in an equivalent amount of lysate.

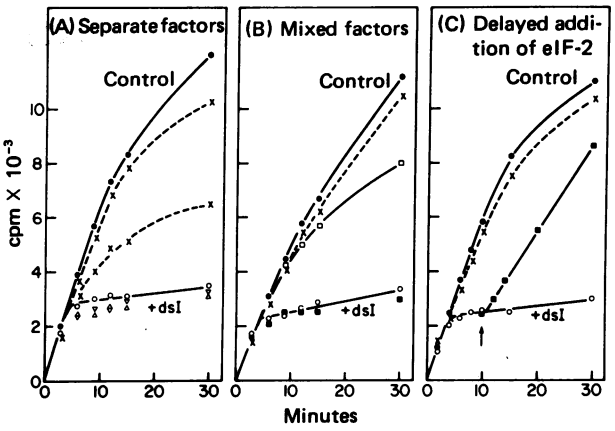

FIG. 4. Effect of initiation factors on inhibition by dsI(r). All protein synthesis assays (25 μl) contained 4 μl dsI(r) (0.07 A_{260} unit) and poly(I·C) (20 μg/ml) except where noted. Initiation factors were added at 0 min as follows: (*A*) ×, 0.6 and 1.2 μg of eIF-2; ▽, 1 μg eIF-1, 0.4 μg eIF-4A, or 0.15 μg eIF-4C + 5; △, 1 μg eIF-4B or 0.8 μg eIF-3; ○, plus dsI(r), minus factors; ●, minus dsI(r), minus factors. (*B*) ×, 1.2 μg eIF-2; ■, 0.1–0.5 μg each of eIF-1, 3, 4A, 4C, 5; □, same amounts of eIF-1, 2, 3, 4A, 4C, 5 combined; ○, plus dsI(r), minus factors; ■, minus dsI(r), minus factors. (*C*) 1.2 μg eIF-2 added at 0 min (×) and at 10 min (■, arrow); ○, plus dsI(r), minus factors; ●, minus dsI(r), minus factors. Incubation was at 30°; at intervals, 3-μl aliquots were assayed for protein synthesis.

Effect of Initiation Factors on the Inhibition by dsI. The inhibition of protein synthesis by dsI(r) was prevented by eIF-2 but not by eIF-1, 3, 4A, 4B, 4C, or 5, either separately (Fig. 4A) or together (Fig. 4*b*). When these factors were combined with eIF-2, restoration of synthesis was dependent only on the amount of eIF-2 added (Fig. 4B). When eIF-2 was added after the onset of inhibition, recovery of synthesis was rapid and approached control kinetics (Fig. 4C).

Solubilization of Ribosome-Associated dsI. Treatment of particulate dsI(r) with 0.5 M KCl releases the dsI activity (Fig. 5A). The solubilized dsI [dsI(s)] inhibits protein synthesis with biphasic kinetics; 3 μg of the dsI(s) preparation shuts off protein synthesis in a 25-μl incubation at 3.5 min (Fig. 5B). The inhibition by dsI(s) is readily reversed by 0.6 μg of eIF-2, but not by eIF-3, which potentiates inhibition to some extent (Fig. 5C). As previously demonstrated (10), high levels of cAMP (10 mM) also prevent the inhibitions induced by dsI(r) or dsI(s) (not shown). It is of interest that the KCl treatment does not appear to dissociate the mycophage dsRNA moiety from the ribosomes, as suggested by the observation that salt-treated ribosomes inhibited protein synthesis in the absence but not in the presence of poly(I·C) (20 μg/ml) (Fig. 5A). Supporting this observation was the finding that the solubilized dsI(s) preparation contained little or no dsRNA, as indicated by the lack of any further inhibitor activation when poly(I·C) was omitted from the incubation (Fig. 5B). These results, although not conclusive, suggest that (*a*) during the activation of dsI, the dsRNA binds tightly to the ribosomes; and (*b*) the dsRNA that remains bound after KCl extraction retains the capacity to induce the formation of dsI in fresh lysates.

Protein Kinase Activities Associated with dsI. Fig. 6 shows the protein kinase activities associated with the activation of dsI on crude ribosomes, as well as with preformed dsI(r), dsI(s), and a partially purified preparation of HRI (see Fig. 3). The incubation of crude ribosomes with mycophage dsRNA (20 ng/ml) stimulates the phosphorylation of the 38,000-dalton polypeptide of added eIF-2 (track 4) compared to a control lacking dsRNA (track 3). A second dsRNA-dependent phos-

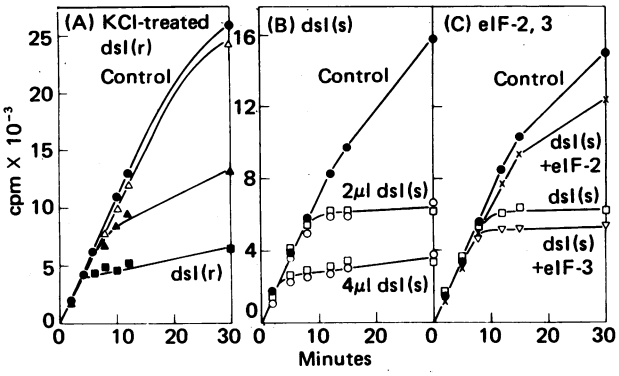

FIG. 5. Solubilization of dsI(r). (*A*) Extraction of dsI(r) with 0.5 M KCl, recovery of dsI(s), and resuspension of the extracted pellets are described in *Methods and Materials*. Protein synthesis assays (50 μl) contained 6 μl of dsI(r) (0.11 A_{260} unit) plus poly(I·C) (20 μg/ml) (■); 0.09 A_{260} unit of resuspended KCl-extracted pellet plus (△) and minus (▲) poly(I·C) (20 μg/ml); ●, 0.12 A_{260} unit of a control ribosomal preparation (−dsI). Incubation was at 30°; at intervals, 5-μl aliquots were assayed for protein synthesis. (*B*) Two microliters (3 μg) and 4 μl (6 μg) of dsI(s) were titrated in protein synthesis assays (25 μl) in the presence (□) and absence (○) of poly(I·C) (20 μg/ml); 5 μg of a control ribosomal extract (−dsI) similarly prepared was assayed in the absence of poly(I·C) (●). At intervals, 3-μl aliquots were assayed for protein synthesis. (*C*) Three micrograms of dsI(s) (□) was assayed in protein synthesis assays (25 μl) in the presence of 0.6 μg of eIF-2 (×) or 1.6 μg of eIF-3 (▽); 4 μg of a control extract (●, see *B*) was similarly assayed. At intervals, 3-μl aliquots were assayed for protein synthesis.

phorylated component appears as a 67,000-dalton band in the absence (track 2) or presence of added eIF-2 (track 4) or added eIF-3 (24) (track 21). This phosphorylation pattern is similar to that previously described by Farrell *et al.* (10) for dsRNA-treated crude reticulocyte ribosomes. It also resembles the phosphorylation profile obtained in dsRNA-treated extracts of mouse fibroblasts (21, 22) and Ehrlich ascites tumor cells (23) sensitized by pretreatment of the intact cells with interferon. The phosphorylation of eIF-2 by ribosome-associated dsI(r) (track 6), dsI(s) (track 9) or partially purified HRI (track 11) is not accompanied by any significant phosphorylation of the 67,000-dalton polypeptide. Although the function of this component is unknown, Farrell *et al.* (10) have proposed that it is involved in inhibitor activation, and that its phosphorylation could account for the ATP requirement during dsI activation. In this regard, recent studies with extracts from interferon-treated cells suggest that dsRNA-induced inhibitions of protein synthesis also involve the dsRNA-dependent formation from ATP of a low molecular weight inhibitor that is capable of inhibiting protein synthesis in lysates in the absence of dsRNA (29, 30).

Fig. 6 also demonstrates that uninduced control ribosomes (−dsI) contain protein kinase activities that phosphorylate the middle polypeptide (49,000 daltons) of eIF-2 (tracks 3, 16) and several subunits of eIF-3 (160,000, 125,000, 65,000 daltons) (tracks 20, 29). These activities, which are unaffected by dsRNA, are also extracted by high KCl treatment (tracks 9, 18, 27); but in a bound or soluble form, these protein kinase activities are not inhibitory (see Fig. 5). These same activities are present in dsI(r) preparations (tracks 6, 23), which also contain the inhibitory protein kinase activity specific for the 38,000-dalton component of eIF-2 (track 6). All of these protein kinase activities are cAMP independent. After treatment of dsI(r) with 0.5 M KCl, the extracted ribosomes display diminished kinase activities toward all of these sites (tracks 14, 25), whereas the KCl extract supernate [dsI(s)] contains all of the kinase activities (tracks 9, 27). By comparison, a similar KCl extract of control ribosomes (−dsI) has no activity towards the 38,000-dalton component of eIF-2 (track 18), but contains the other protein kinase activities (data for eIF-3 not shown). The solubilized dsI(s) also expresses several additional endophosphorylation activities, including phosphorylation of a 65,000-dalton polypeptide (tracks 8, 9, 26), which appears to coincide with a similar phosphorylated band of eIF-3 (tracks 20, 23, 27, 29); however, its function and identity are not resolved.

Control ribosomal preparations (−dsI) and untreated crude ribosomes display a low level dsRNA-independent phospho-

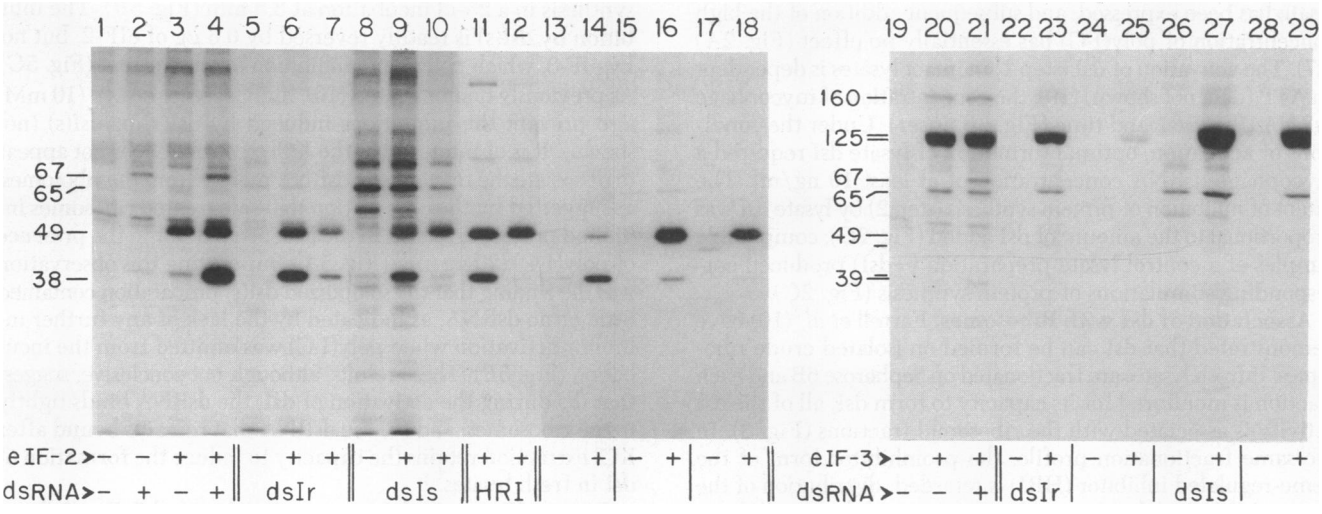

FIG. 6. Protein kinase activities associated with dsI activation, dsI(r), and dsI(s). Protein kinase assays (20 μl), electrophoresis in sodium dodecyl sulfate/acrylamide, and autoradiography are described in *Methods and Materials*. eIF-2 (0.6 μg), eIF-3 (1.6 μg), and mycophage dsRNA (20 ng/ml) were added where indicated. Other additions are: 0.07 A_{260} unit of crude ribosomes (tracks 1–4, 19–21); 0.04 A_{260} unit of dsI(r) (tracks 5–7, 22, 23); 3 μg of dsI(s) (tracks 8–10, 26, 27); 1.8 μg of *N*-ethylmaleimide-activated partially purified HRI (tracks 11, 12) (see Fig. 3); 0.03 A_{260} unit of dsI(r) ribosomes after KCl extraction (tracks 13, 14, 24, 25); 0.04 A_{260} unit of a control ribosome preparation (−dsI) (tracks 15, 16, 28, 29); 4 μg of a KCl extract of the control ribosome preparation (−dsI) (slots 17, 18); and 30 μM hemin (slots 7, 10, 12). eIF-2 subunits migrated as bands of 52,000, 49,000, and 38,000 daltons; eIF-3 subunits migrated as bands of 160,000, 120,000–125,000, 65,000, 49,000, 45,000, 41,000, and 39,000 daltons.

rylation of the 38,000-dalton polypeptide of eIF-2 (tracks 3, 16). Because these preparations are not inhibitory, this limiting activity probably reflects a low level activity normally present in lysates, although the possibility cannot be excluded that this limited activation is a result of the processing procedures. Finally, it should be noted that under the conditions of incubation, the addition of hemin (30 μM) selectively diminishes the phosphorylation of the 38,000-dalton polypeptide of eIF-2 by dsI(r) (track 7), dsI(s) (track 10), and partially purified HRI (track 12), compared to the effect of hemin on the phosphorylation of the 49,000-dalton polypeptide of eIF-2 by these same preparations. Because these inhibitors are active in hemin-supplemented lysates, the significance of the selective effects of hemin on these protein kinase activities remains to be defined.

As in the case of heme deficiency, and in accord with the findings of Farrell *et al.* (10), these observations demonstrate a correlation between the activation of a protein kinase activity that phosphorylates eIF-2 and the inhibition of protein synthesis initiation in reticulocyte lysates. Among several unresolved questions are the specific role of dsRNA in the mechanism of inhibitor activation and the relationship between the two protein kinase activites induced by heme deficiency and dsRNA.

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